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Phytol and Peroxisome Proliferation

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ABSTRACT. Infantile Refsum's disease is characterized by high levels of phytanic acid and the absence of normal hepatic peroxisomes. We investigated the *in vivo* influence of phytol, a precursor of phytanic acid, on peroxisomes by both biochemical and morphological methods. Enhanced supply proliferation of . The peroxisomal β-oxidizing capacity as as exchanges of acyl moieties between feeding peroxisome proliferation in duodenal epithelium, in myocardium and in skin sebaceous glands, but not in kidney. (*Pediatr Res* 20: 411–415, 1986)

Phytanic acid is a minor lipid component of normal human serum. In patients with adult-onset Refsum's disease this fatty acid accumulates in the lipids of several tissues (1, 2). The metabolic defect resulting from an impairment of the α-oxidation system is clinically revealed by crisis occuring in the course of nutritional overload by phytanic acid (2–4). Phytanic acid and its precursor phytol are present in most diets (5). In humans and in the adult Refsum patients (2). After only 2 days of a 2% phytol diet mouse liver contains significant amounts of phytanic acid and its metabolites, whereas in control livers these acids were not detectable (12).

patients (17, 18) indicate a link with two other neonatal "peroxisomal diseases," Zellweger's cerebrohepatorenal syndrome and adrenoleukodystrophy (autosomic recessive), in which peroxisomes are either absent or lacking several functions (19–24). The ultrastructural and biological findings reported in infantile Refsum's disease have not yet been examined in adult-onset Refsum's disease; peroxisomes were studied in cultured fibroblasts from adult-onset Refsum patients, but not in other cell types (24). It was recently demonstrated that certain metabolites (hypoglycin) can have a destructive effect on rat liver peroxisomes (25). We now investigate the effect of phytanic acid accumulation following enhanced dietary supply of phytol on peroxisomal abundance and enzymology in several organs from mice. We also discuss the possible role of peroxisomes in the catabolism of phytanic acid.

MATERIALS AND METHODS

control mice received meal + 10% soya oil (w/w). Phytol (95%) was obtained from Serva Feinbiochemica, Heidelberg, Germany). Diets were given during 3 to 21 days. Before sacrifice

Each experimental group consisted of at least four animals. All results are presented as the mean ± SEM. For statistical

RESULTS

After 3 days of phytol diet the catalase activity of the liver has significantly increased (Table 1). A phytol dose of 0.05% is not effective; a large dosis of 5% is only slightly more effective than a 0.5% dosis and provokes serious distress in the animals. For the last reason the dosis of 0.5% phytol was chosen for further experiments. After 11 days of this diet, liver catalase activity is increased; this increase is not more pronounced than after 3 days. The increase of liver catalase activity is present in both male and female animals.

The influence of a 0.5% phytol diet during 11 days on seven liver enzyme activities is summarized in Table 2. The peroxisomal β-oxidation specifically measured by its first step is increased more than 5-fold, while two other peroxisomal enzymes, L-α-hydroxyacid oxidase type A and urate oxidase keep their normal activity. Peroxisomal carnitine octanoyltransferase and total mitochondrial carnitine palmitoyltransferase also display more activity as does total liver carnitine acetyltransferase. Butyryl-CoA dehydrogenase, a marker enzyme of the mitochondrial matrix, was also assayed and its activity was found to be 1.5-fold higher in phytol-treated than in control liver.

Serum cholesterol levels are not decreased after 21 days of a 0.5% phytol diet; triglyceride levels on the contrary decrease significantly during this period (Table 3).

Liver sections of 20 μm show a marked increase in catalase staining after 0.5 and 5% phytol for 3 and 11 days, but not after 0.05% phytol, when compared to control animals. One-μm Epon sections give evidence that the number of peroxisomes is raised by feeding 0.5 and 5% phytol (Fig. 1), and not raised by 0.05%. Individual peroxisomes also appear larger and more darkly stained than in controls. A difference between 0.5 and 5% is visible. Proliferation and enlargement of peroxisomes is confirmed by electron microscopy (Fig. 2). The subcellular organelles are otherwise normally shaped.

No peroxisomes are visible by light microscopy in 4-μm Epon sections of duodenum in control and 0.5% phytol-fed mice. Peroxisome proliferation is noticed in the duodenal epithelial cells of mice fed 5% phytol during 5 days. This proliferation is most pronounced at the base of the villi (Fig. 3 *a* and *b*).

In skin sebaceous glands of 5% phytol-fed mice (5 days) peroxisomes are visible in Epon sections (Fig. 3 *c* and *d*). This is not the case in the other groups.

In mouse myocardium light microscopy shows no visible peroxisomes in control animals. In 5% phytol treated mice peroxisomes are present (Fig. 3 *e* and *f*).

Peroxisome proliferation in liver, duodenum, skin sebaceous glands, and myocardium is observed in male and female mice.

Cryostat sections of kidneys show no difference between control and phytol-fed animals. In adrenal glands very few and small peroxisomes are seen both in controls and in treated mice.

It seems important to stress the toxicity of phytol to animals (6, 35, 36). Mice fed with 0.5% phytol did not show any signs of distress. Mice fed a 5% phytol diet became ill sometimes after 3 or 4 days; they lose appetite, start shivering, and show the changes in the skin described by Klenk and Kremer (6).

Table 1. *Influence of phytol on mouse liver catalase activity after 3 and 11 days of phytol diet**

Conditions	Catalase (U_M/g of liver)	Ratio
Controls	90 ± 1	
3 days of phytol diet		
0.05%, male	98 ± 4	1.09
0.5%, male	163 ± 3	1.81
5%, male	178 ± 5	1.98
0.5%, female	140 ± 5	1.56
5%, female	174 ± 5	1.93
11 days of phytol diet		
0.5%, male	138 ± 6	1.53

* All phytol influenced catalase values are significantly different from controls ($p < 0.01$) except the 0.05% value.

Table 2. *Influence of 11 days of a 0.5% phytol diet on liver enzymes in adult male mice**

Enzymes	Controls	0.5% Phytol fed	Ratio
Palmitoyl-CoA oxidase	522 ± 31	2666 ± 319	5.11†
L-α-Hydroxyacid oxidase (type A)	278 ± 31	281 ± 22	1.01
Urate oxidase	737 ± 95	665 ± 34	0.90
Carnitine palmitoyltransferase	1231 ± 66	1856 ± 159	1.51†
Carnitine octanoyltransferase	2306 ± 87	5044 ± 259	2.19†
Carnitine acetyltransferase	239 ± 41	680 ± 92	2.85†
Butyryl-CoA dehydrogenase	731 ± 28	1063 ± 23	1.45†

* Enzyme activities are expressed as nmol of substrate consumed or product formed per min and per g of liver.

† $p < 0.01$.

Table 3. *Influence of phytol diet on adult male mouse serum cholesterol and triglyceride levels*

Conditions	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Controls	120 ± 5	115 ± 10
3 days of phytol diet		
0.05%	139 ± 4	108 ± 14
0.5%	108 ± 5	120 ± 22
11 days of phytol diet		
0.5%	106 ± 10	145 ± 30
21 days of phytol diet		
0.5%	118 ± 8	70 ± 8*

* $p < 0.01$.

DISCUSSION

Children with infantile Refsum's disease possess abnormal microbodies without catalase, or no microbodies at all (15, 16). Whereas in the adult form of Refsum's disease the localization of the enzyme defect, *i.e.* phytanic acid oxidase deficiency, is well established, reasons for impaired metabolism of phytanic acid in the infantile form remain to be elucidated. Selective accumulation of one or several intermediates of phytanic acid catabolism in infantile Refsum's disease cannot be definitely ruled out. As in the case of hypoglycin (25), such metabolites might be toxic for peroxisomes. Overload of the entire set of reactions leading to phytanic acid breakdown has been achieved by exogenous administration of high doses of phytol to mice. Our experiments show that enhanced supply of phytol in the diet of mice is not destructive to peroxisomes. Actually, the number and size of peroxisomes is increased in liver and in several other organs, with a cytochemical picture contrasting to the situation observed in liver from infantile Refsum patients [15, 16].

We also demonstrate that phytol feeding (0.5%) increases the activity of peroxisomal β-oxidation. It has no effect on other peroxisomal marker enzymes. Carnitine acyltransferases involved in fatty acid metabolism show increased activity but less than the peroxisomal fatty acyl-CoA oxidase. Although both peroxisome proliferators are chemically very different, the parallel which can be drawn between the effects of phytol and clofibrate on the enzymes investigated is striking: stimulation of peroxisomal β-oxidation and increased capacity to metabolise

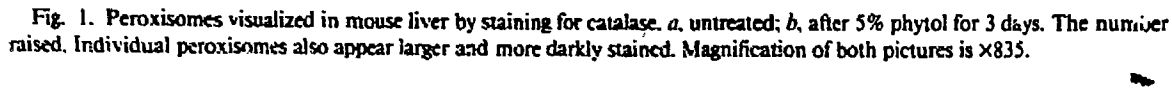

Fig. 1. Peroxisomes visualized in mouse liver by staining for catalase. *a*, untreated; *b*, after 5% phytol for 3 days. The number of peroxisomes is raised. Individual peroxisomes also appear larger and more darkly stained. Magnification of both pictures is ×835.

Fig. 2. Electron micrograph of mouse liver stained for visualization of peroxisomes. *a*, untreated; *b*, after 11 days of 5% phytol. Peroxisomal number as well as size are increased. Magnification of both pictures is ×5500.

products of this oxidation (*e.g.* octanoyl-CoA) (37) consisting in stimulation of exchanges between peroxisomes and mitochondria (augmented carnitine acyltransferase activities) beside higher ability of mitochondria to catabolise short-chain fatty acids (increased butyryl-CoA dehydrogenase activity).

The significant decrease of triglyceride levels between 11 and 21 days of phytol diet is an observation for which an appropriate explanation is not available. There obviously is a shift in time between the hypotriglyceridemic effect and peroxisome proliferation. Examples of uncoupling of these phenomena are known in the literature (38).

Do our results provide any clue in the nature of the peroxisomal enzymes implicated in the metabolism of phytanic acid, a C-20 branched fatty acid? Phytanic acid oxidase, catalyzing the α-oxidation step, has been described to be present in or linked with mitochondrial fractions (39, 40). However, in these studies peroxisomes contaminating such fractions were not considered. Other investigators suggested that the peroxisomal α-hydroxyacid oxidase could take part in phytanic acid α-oxidation (24). Our experiments show no induction of this enzyme following the phytol diet. After the initial α-oxidation step, phytanic acid is subject to normal β-oxidation (2). Examples are known in which peroxisomes and peroxisomal enzymes are induced by their substrates or metabolic conditions requiring their contribution, *e.g.* the induction of hepatic peroxisomal β-oxidation by feeding high fat diets in rats (41, 42) and peroxisomal proliferation in brown adipose tissue by cold stress (43). Proliferation of peroxisomes and the considerable and selective induction of peroxiso-

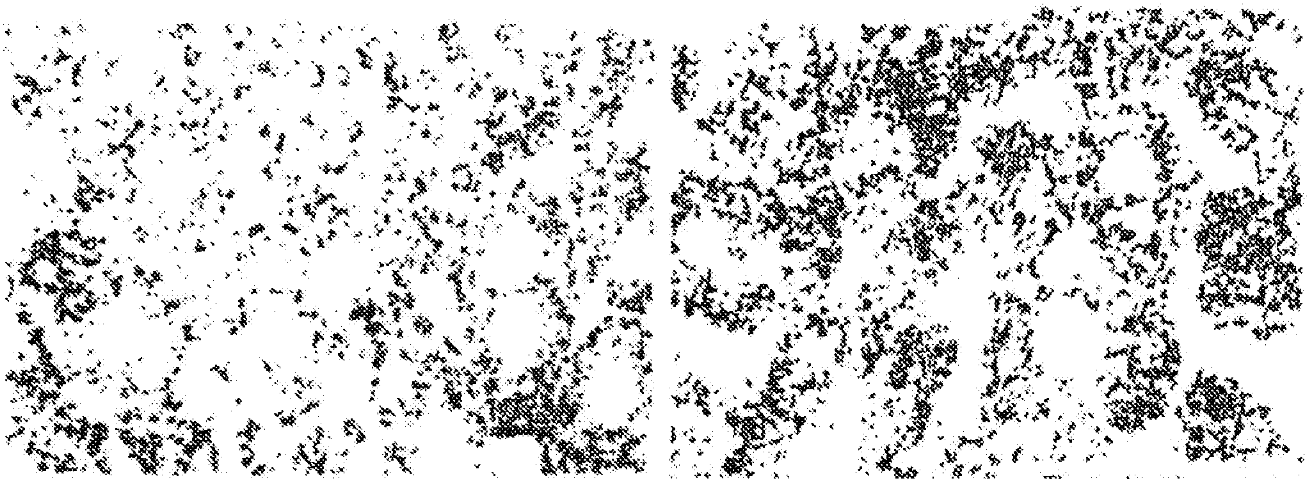

Fig. 1. Peroxisomes [illegible] showing [illegible] 5% [illegible] days. The number of peroxisomes is [illegible]. Individual peroxisomes [illegible]. Magnification of both pictures [illegible].

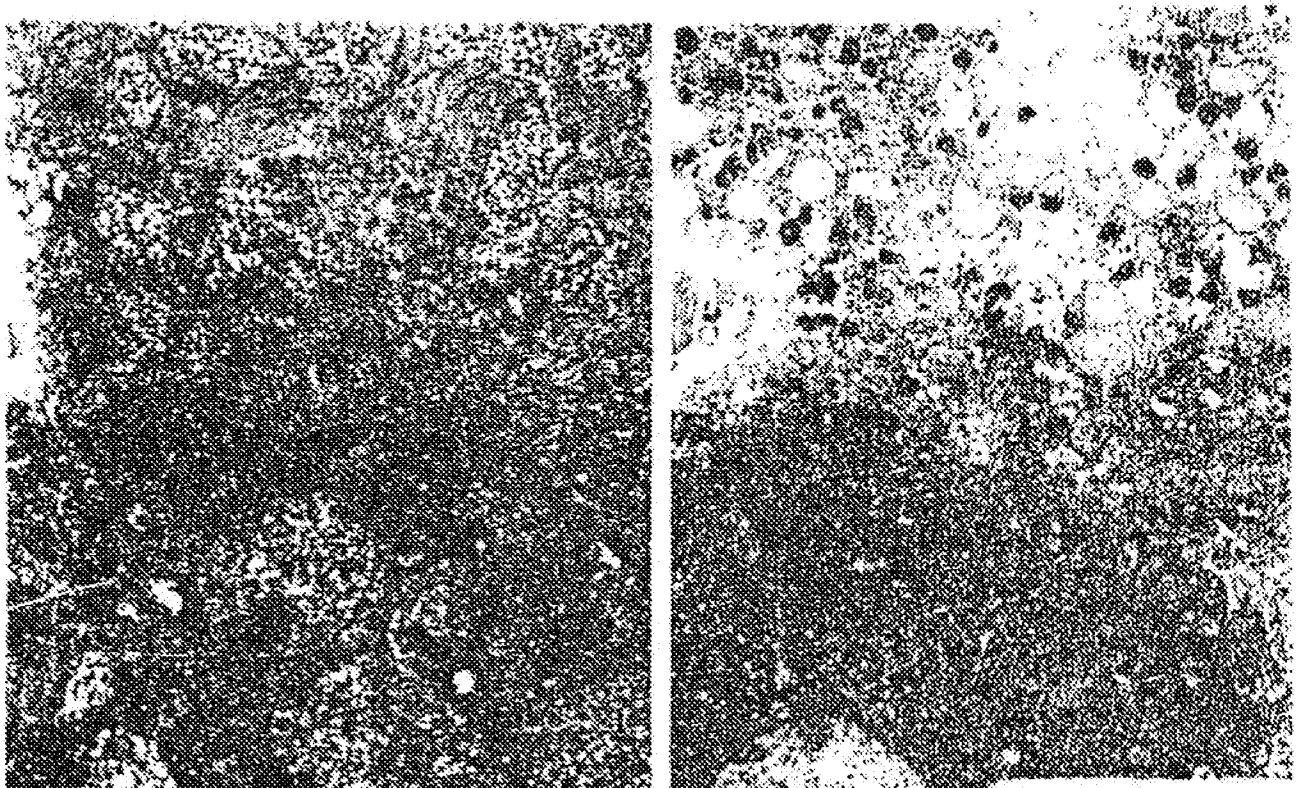

Fig. 2. Electron micrographs of mouse liver stained for [illegible] of peroxisomes (a) untreated; b) after 11 days of 5% phytol. Peroxisomal number as well as size are increased. Magnification of both pictures [illegible].

products of this oxidation are octanoyl-CoA (37) consisting in stimulation of exchanges between peroxisomes and mitochondria (augmented carnitine acyltransferase activity) beside higher ability of mitochondria to catabolise short-chain fatty acids (increased butyryl-CoA dehydrogenase activity).

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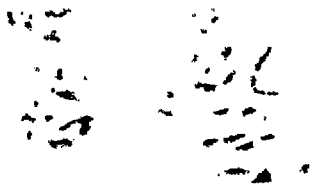

Fig. 3. Cytochemical staining for catalase of mouse duodenum (*a*, *b*), cutaneous sebaceous gland (*c*, *d*), and myocardium (*e*, *f*). By light microscopy no peroxisomes are recognized in untreated animals (*a*, *c*, *e*). After 5% phytol feeding for 5 days (*b*, *d*, *f*) peroxisomes become conspicuous in all three organs. Magnification of all the pictures is ×1300 (phase contrast).

mal β-oxidation enzymes after phytol feeding suggest a role for peroxisomes in the latter metabolic phase of phytanic acid breakdown. For this reason, the absence of normal peroxisomes in infantile Refsum's disease might explain the accumulation of phytanic acid, because our results demonstrate that the opposite (destruction of peroxisomes by phytanic acid) does not occur.

It is striking that the phytol effect is selective for some cell types, while in others no change is visible by light microscopy. Our experiments show peroxisome proliferation following phytol diet in liver, duodenal epithelium, myocardium, and skin sebaceous glands, but not in kidneys and adrenal glands. Accumulation of phytanic acid in several body tissues was studied and

Fig. 3. Cytochemical staining for catalase of mouse [illegible] (a, b), [illegible] cutaneous sebaceous gland (c, d) and myocardium (e, f). By light microscopy peroxisomes are [illegible] in untreated animals (a, c, e). After [illegible] phytol feeding for 5 days (b, d, f) peroxisomes become conspicuous in all three organs. Magnification [illegible]. All pictures [illegible] phase contrast.

[illegible] glands, but not in kidneys and adrenal glands. Accumulation of phytanic acid in several body tissues was studied and

relationship is missing in the kidney. Renal proximal tubular epithelium normally contains numerous large peroxisomes. In Zellweger's cerebrohepatorenal syndrome peroxisomes are absent in kidneys as well as in liver. No data are available about

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